

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor: Tariq M. RANA
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Title : DELIVERY OF siRNAs
Attorney Docket No. : 20336-00016
Examiner : Kimberly Chong
Group Art Unit : 1635

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SECOND DECLARATION OF TARIQ M. RANA UNDER 37 C.F.R. §1.132

I, Tariq M. Rana, hereby make the following declaration:

1. I am the inventor of the invention described in U.S. patent application 10/722,176 filed November 24, 2003 ("the '176 application"), and U.S. provisional patent application 60/430,520, filed on November 26, 2002 ("the '520 application").

2. The '176 application as filed (and published as US 2004/0204377 A1 on October 12, 2004) describes the use of a delivery mixture comprising a delivery agent consisting of a dendrimer mixed with a nucleic acid capable of mediating RNA interference in Example 1 (paragraph [0102] of the published application and FIGS. 1A and 1B), Example 2 (paragraph [0103] of the published application and FIG. 2), and Example 7 (paragraph [0111] of the published application and FIGS. 9A –9I).

3. The corresponding description of the use of a delivery mixture comprising a delivery agent consisting of a dendrimer mixed with a nucleic acid capable of mediating RNA interference can be found in U.S. provisional patent application no. 60/430,520 in Example 2 (page 19 line 23 to page 20, line 31 and FIGS. 1A and 1B), Example 3 (page 21, lines 1-21 and FIG. 2), and Example 8 (page 24, line 29 to page 25, line 14 and FIGS. 9A –9I).

4. Experiments corresponding to the description of the use of a delivery mixture comprising a delivery agent consisting of a dendrimer mixed with a nucleic acid capable of mediating RNA interference were carried out in my laboratory by Ya-Lin Chiu under my direction and supervision.

5. Exhibits 1-11 are copies of notebook entries from the laboratory notebook of Ya-Lin Chiu, titled "Delivery Method and Localization of siRNA," for the period October 10, 2002 through February 11, 2003. Exhibits 1-11 are notebook entries of experiments carried out during the period October 21, 2002 through November 19, 2002.

a. Exhibit 1 is an outline of experimental protocol used in experiments dated 10/21/02 – 10/25/02 for determination of transfection efficiency of siRNA by various delivery agents, including PAMAM dendrimer and lipofectamine.

b. Exhibit 2 is a description of the PAMAM dendrimer used in experiments. A PAMAM G4 dendrimer in a 90 µg/µl methanol solution was used.

c. Exhibit 3 is fluorescence level results of siRNA transfection experiments carried out 10/23/02.

d. Exhibit 4 is a computer printout of fluorescence results of transfection experiments using PAMAM G4 dendrimer and Lipofectamine, and carried out 10/23/02.

e. Exhibit 5 is graph results of transfection experiments introducing Cy3-labeled EGFP duplex siRNAs using PAMAM G4 dendrimer and Lipofectamine carried out 10/23/02. A note in the page indicates the product code number in the Aldrich catalog for the PAMAM Generation 4 dendrimer used in experiments. Exhibit 8 is a graph summary of results of similar transfection experiments introducing Cy3-labeled EGFP duplex siRNAs carried out 10/23/02.

f. Exhibit 6 is protein levels of lysates from cells transfected with CDK9 siRNA by PAMAM dendrimer 10/29/02.

g. Exhibit 7 is immunoblot results of lysates from cells transfected with CDK9 siRNA by PAMAM dendrimer. Exhibit 9 is also immunoblot results of lysates from cells transfected with CDK9 siRNA by PAMAM dendrimer on 10/25/02.

h. Exhibit 10 and Exhibit 11 depict results of microscopic examination of HeLa cells transfected with Cy3-SS/AS siRNA by Lipofectamine or PAMAM, respectively on 11/19/02.

6. The notebook pages of Exhibit 2 and Exhibit 5 evidence use of generation 4 PAMAM dendrimer for transfection experiments. Each of the experiments carried out during the period 10/21/02 through 11/19/02 using a PAMAM dendrimer as a delivery agent used generation 4 PAMAM dendrimer.

7. Exhibits 1-11 are experiments and results corresponding to Example 1 (paragraph [0102] of the published application and FIGS. 1A and 1B), Example 2 (paragraph [0103] of the published application and FIG. 2), and Example 7 (paragraph [0111] of the published application and FIGS. 9A –9I) of the '176 application.

a. Example 1 and FIG. 1A and FIG. 1B correspond to the experiments and results depicted in Exhibits 1, 3, 4, 5, and 8.

b. Example 2 and FIG. 2 correspond to the experiments and results in Exhibits 6, 7, and 9.

c. Example 7 and FIG. 9 correspond to the experiments and results in Exhibit 11.

8. Exhibits 1-11 are experiments and results corresponding to Example 2 (page 19 line 23 to page 20, line 31 and FIGS. 1A and 1B), Example 3 (page 21, lines 1-21 and FIG. 2), and Example 8 (page 24, line 29 to page 25, line 14 and FIGS. 9A –9I) of U.S. provisional patent application no. 60/430,520.

a. Example 2 and FIG. 1A and FIG. 1B correspond to the experiments and results depicted in Exhibits 1, 3, 4, 5, and 8.

b. Example 3 and FIG. 2 correspond to the experiments and results in Exhibits 6, 7, and 9.

c. Example 8 and FIG. 9 correspond to the experiments and results in Exhibit 11.


9. The experiments described in the '176 application (Example 1 (paragraph [0102] of the published application and FIGS. 1A and 1B), Example 2 (paragraph [0103] of the published application and FIG. 2), and Example 7 (paragraph [0111] of the published application and FIGS. 9A –9I) of the '176 application) used a generation 4 PAMAM dendrimer as a delivery agent mixed with a nucleic acid capable of mediating RNA interference.

10. The experiments described in U.S. Provisional application 60/430,520 (Example 2 (page 19 line 23 to page 20, line 31 and FIGS. 1A and 1B), Example 3 (page 21, lines 1-21 and FIG. 2), and Example 8 (page 24, line 29 to page 25, line 14 and FIGS. 9A –9I) of U.S. provisional patent application no. 60/430,520) used a generation 4 PAMAM dendrimer as a delivery agent mixed with a nucleic acid capable of mediating RNA interference.

11. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Respectfully Submitted,

Date: Oct 26, 2007



Tariq M. Rana

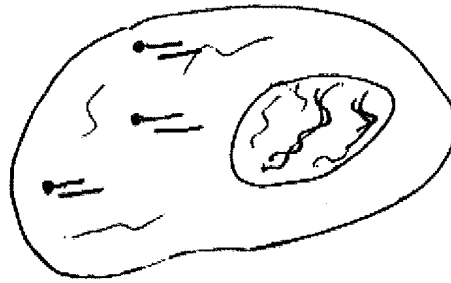
10/21/02 ~ 1022/02

Determine transfection efficiency
Using cy3-labeled siRNA as indicator

EGFP 5'cy3-SS/AS Duplex

- Transfection by
1. Lipofectamine
 2. Nanoparticle #1
 3. PAMAM

Hela cell



on 60mm plates

6hr incubation at 37°C

washed 3x by 5mL PBS

Qiagen DNA/RNA Extraction kit

pellet
protein
Removed by strong denaturing reagent
followed by ϕ 13000rpm 20 min
4°C

solution
containing DNA
RNA
Small RNA

Isopropanol
precipitation
4°C, 13000rpm 30 min

Redissolve pellet in H₂O
(heat at 70°C 10 min)

Detect cy3 signal
by Fluorometer

Ya-Lin Chen

EXHIBIT 1

PM AM - G4

10/21/02

10g/110 mL.

90 ug/ul. in Methanol.

EXHIBIT 2

10/23/02

102302-1

EGFP

18560

2 10690

3 18220

4 19400

5 11650

6 10570

7 8500

8 8867

9 7982

11 11080

11 10780

12 4126

13 5962

14 4698

102302-2

EGFP

RFP 2

2 272300 104300

3 53180 101900

4 252200 98710

5 266600 104300

6 286400 119000

7 364800 160300

8 587700 276300

9 594500 291400

102302-3

10 194700 87680

11 132900 84660

12 89570 86980

13 58490 91380

14 54000 91850

15 35040 92920

16 26180 106900

17 25970 115800

18 28160 135900

19 22080 120500

102302-4

EGFP

RFP

20 262800 97460

21 261400 98950

22 218700 84620

23 200700 84480

24 208600 90110

25 229700 113900

26 188300 108000

27 203800 124400

28 140300 91130

102302-5

29 249200 98710

30 244900 97150

31 246500 104900

32 219900 101800

33 222800 105500

34 193400 94150

35 166800 90910

36 153500 91580

37 128200 92100

See Detail in

Modified siRNA Effect

102302-6

EGFP

RFP

38 257200 111900

39 275500 103900

40 306600 110500

41 240200 83860

42 264900 104100

43 294500 120000

44 230700 95480

45 234900 94740

46 252700 93130

47 221100 81610

48 123300 45220

49 52850x2 18260x2

50 256900 90710

51 203400 66640

52 105300 47270

53 48440x2 14240x2

EXHIBIT 3

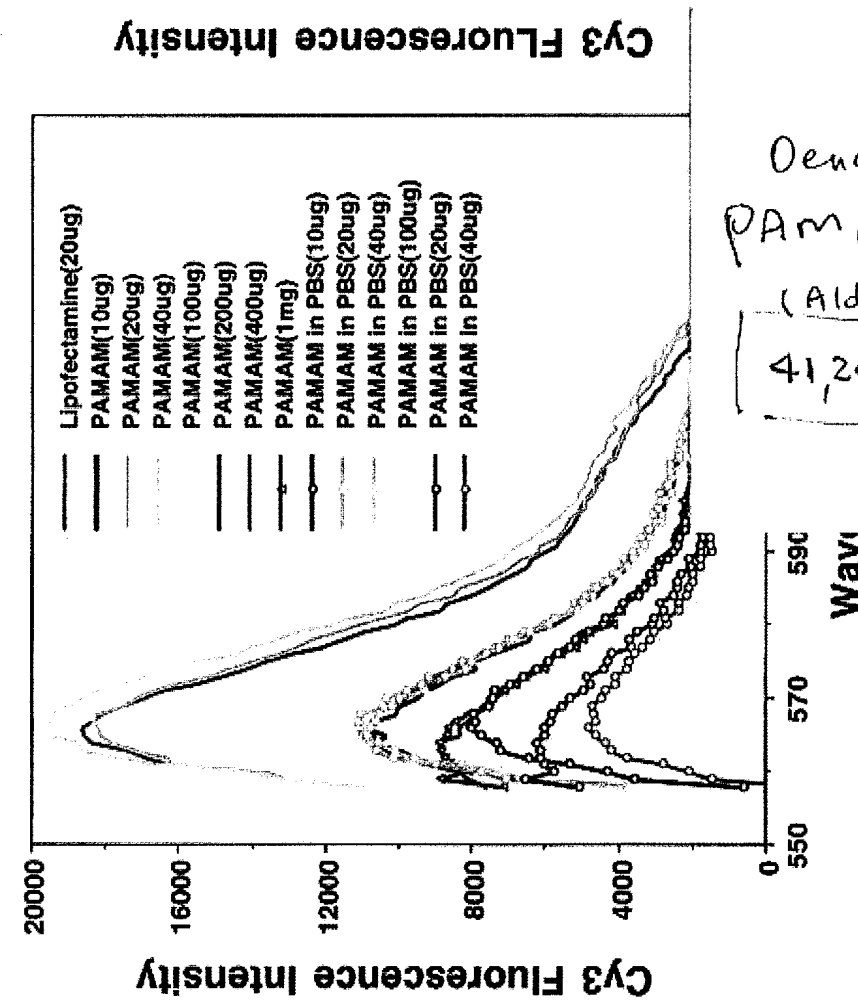
Ya-Lin Chiu

	Cy3 FLuorescence Intensity	Treatment	Relative siRNA uptake Efficiency
1	18560.000	Lipofectamin (20ug)	1.000
2	10690.000	PAMAM(10ug)	0.576
3	18220.000	PAMAM(20ug)	0.982
4	19400.000	PAMAM(40ug)	1.045
5	11650.000	PAMAM(100ug)	0.628
6	10570.000	PAMAM(200ug)	0.570
7	8500.000	PAMAM(400ug)	0.458
8	8867.000	PAMAM(1mg)	0.478
9	7982.000	PAMAM 1 in PBS (10ug)	0.430
10	11080.000	PAMAM 1 in PBS (20ug)	0.597
11	10780.000	PAMAM 1 in PBS (40ug)	0.581
12	4126.000	PAMAM 1 in PBS (100ug)	0.222
13	5962.000	PAMAM 1 in PBS (200ug)	0.321
14	4698.000	PAMAM 1 in PBS (400ug)	0.253

EXHIBIT 4

*Ja-Lin Ch**M*


Comparison Analysis of PAMAM to Lipofectamine (EGFP 5'Cy3-SS/AS siRNA as transfection efficiency indicator)



	OD750	Protein(ug/ul)	60ug	ul/60ug	Buffer
1	0.716	6.616	60.000	9.069	11.931
2	0.613	5.642	60.000	10.634	10.366
3	0.779	7.211	60.000	8.320	12.680
4	0.783	7.249	60.000	8.277	12.723
5	0.648	5.973	60.000	10.045	10.955
6	0.587	5.396	60.000	11.119	9.881
7	0.553	5.075	60.000	11.823	9.177
8	0.471	4.800	60.000	13.955	7.045
9	0.968	3.326	60.000	18.040	2.960

mock (40ug PAMAM only)
100nM cdk9 siRNA by 20ug
40ug
100ug PAMAM-C
200ug
400ug
1 mg

EXHIBIT 6


Yan-Li Chen

10/25/02 ~ 10/30/02

→ From 10/21/02 and 10/25/02 Experiment.

We know that PAMAM Dendrimer can send siRNA into the cell.
but cannot send the reporter plasmid in (size constraint?)
so ⇒ cannot use dual fluorescence assay to get Quantitation data

→ Silencing of cdk9 expression by PAMAM mediated transfection

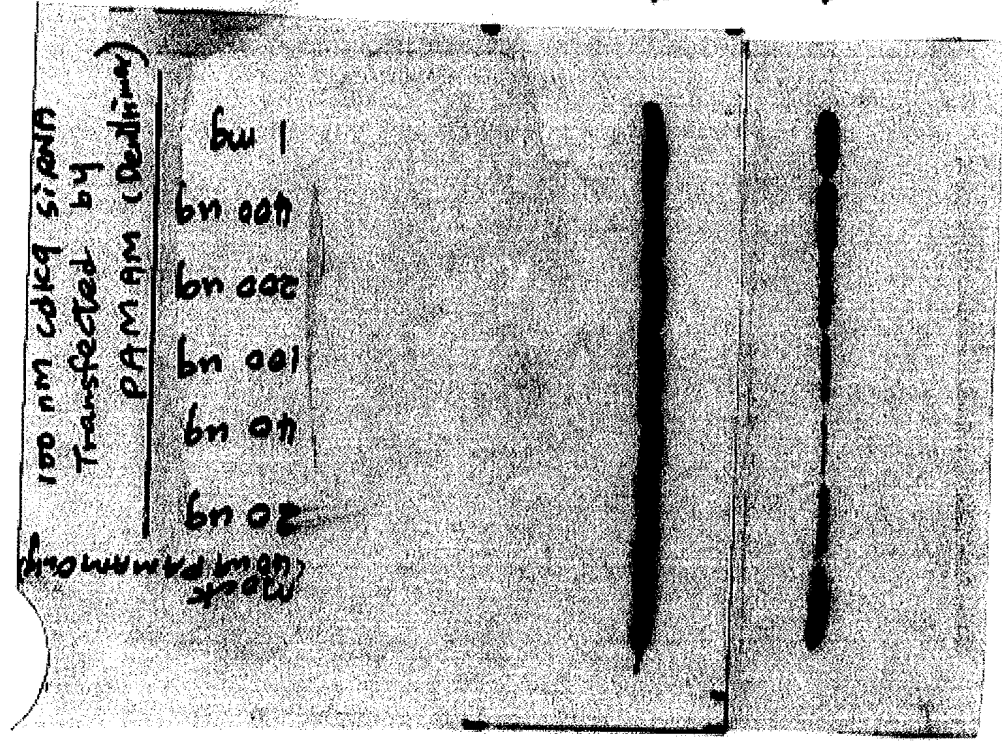
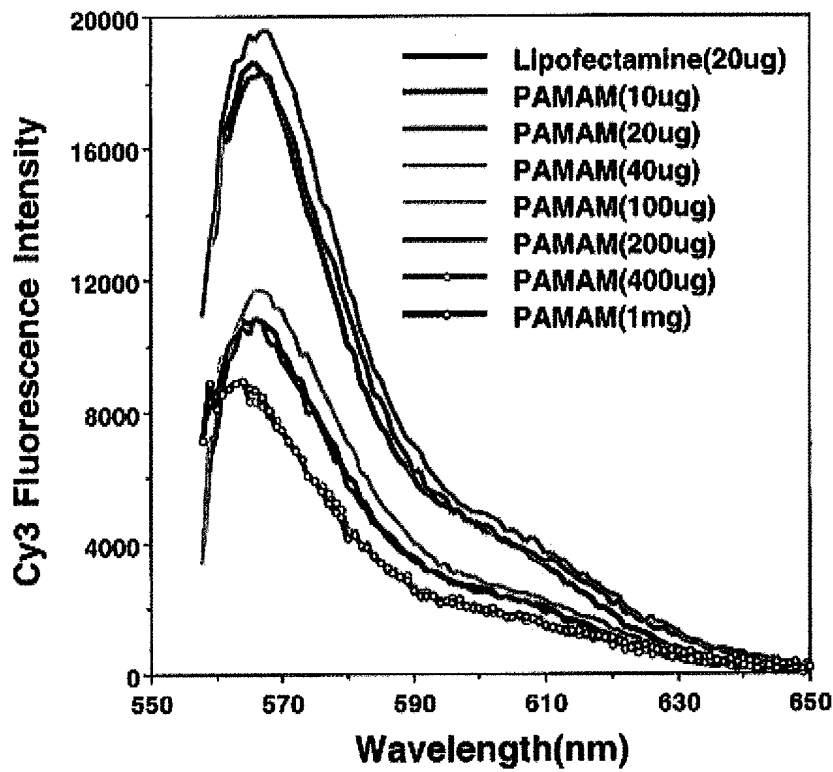


EXHIBIT 7

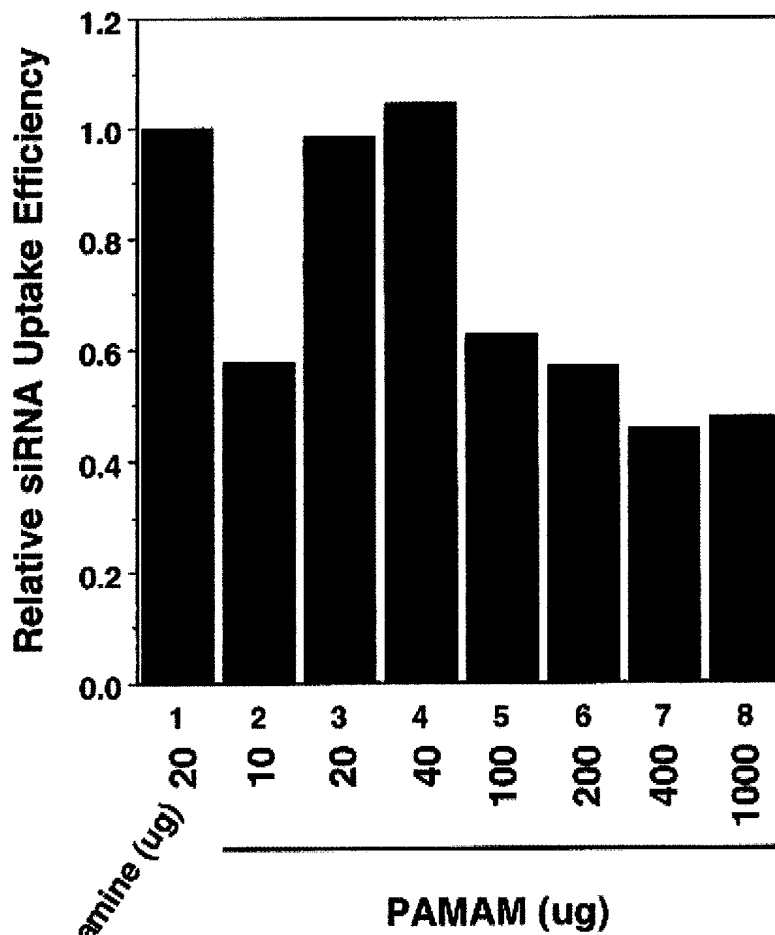
(A)

Fig 1



10/23/2002

(B)



Lipofectamine (ug) 20 10 20 40 100 200 400 1000

EXHIBIT 8

in
Yan-Lin Chen

10/25/2002

Fig 2

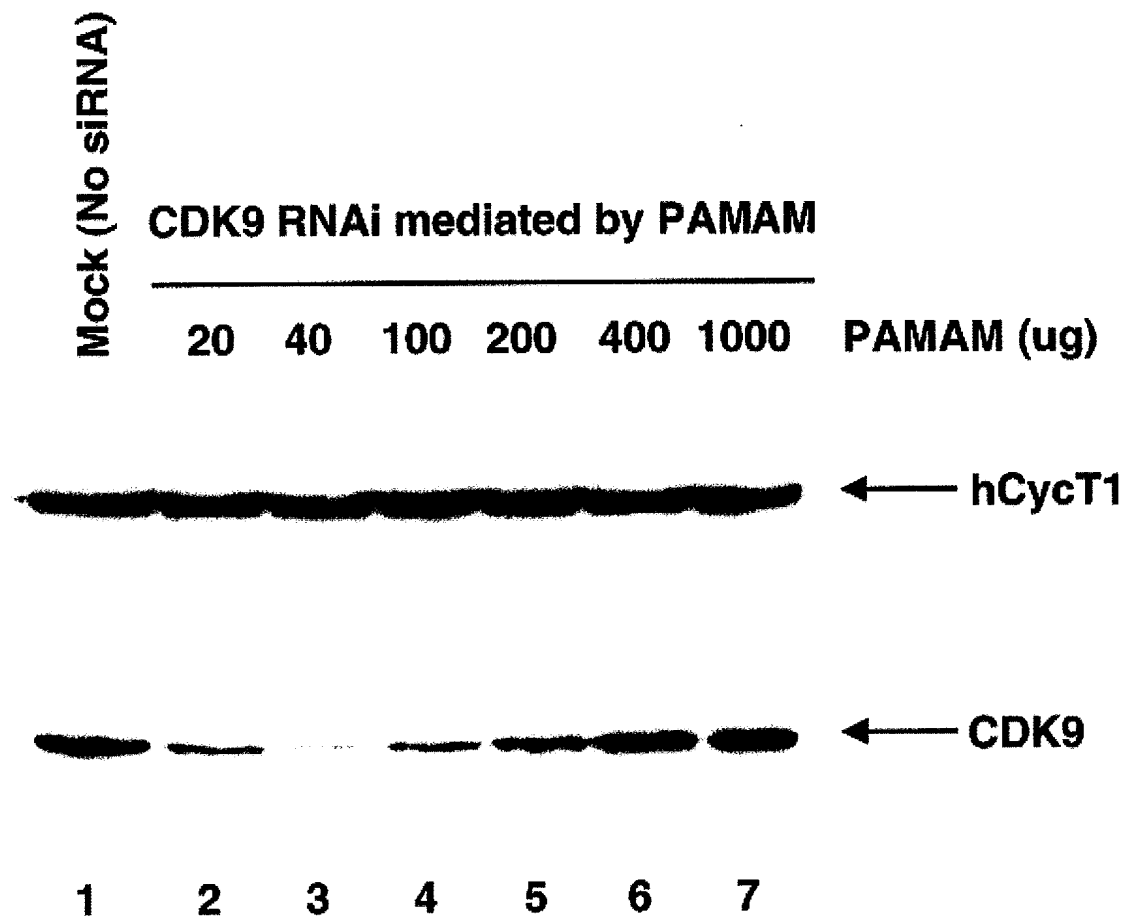
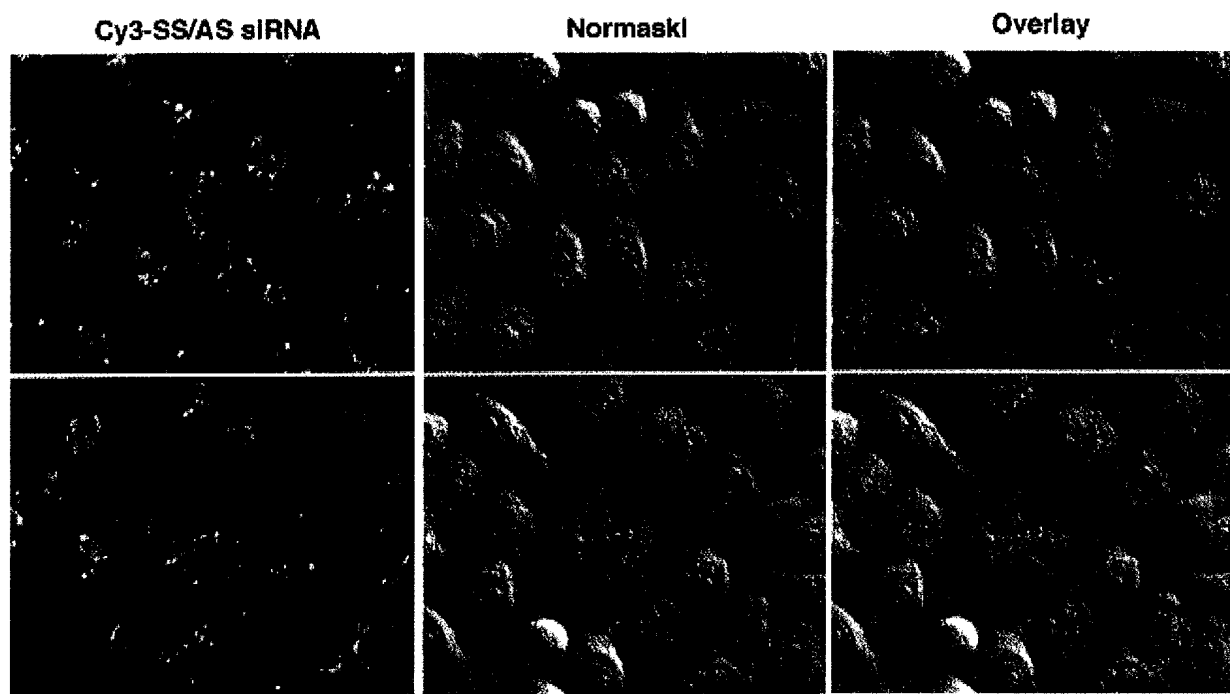


EXHIBIT 9

TL
Ya-Li chin

11/19/02

Uptake of siRNA by HeLa Cells (20ug/1mL Lipofectamine-Mediated Transfection, 6h)(111902)



400X, 35 mm dish with coverslip bottom

Lelco Demo

EXHIBIT 10

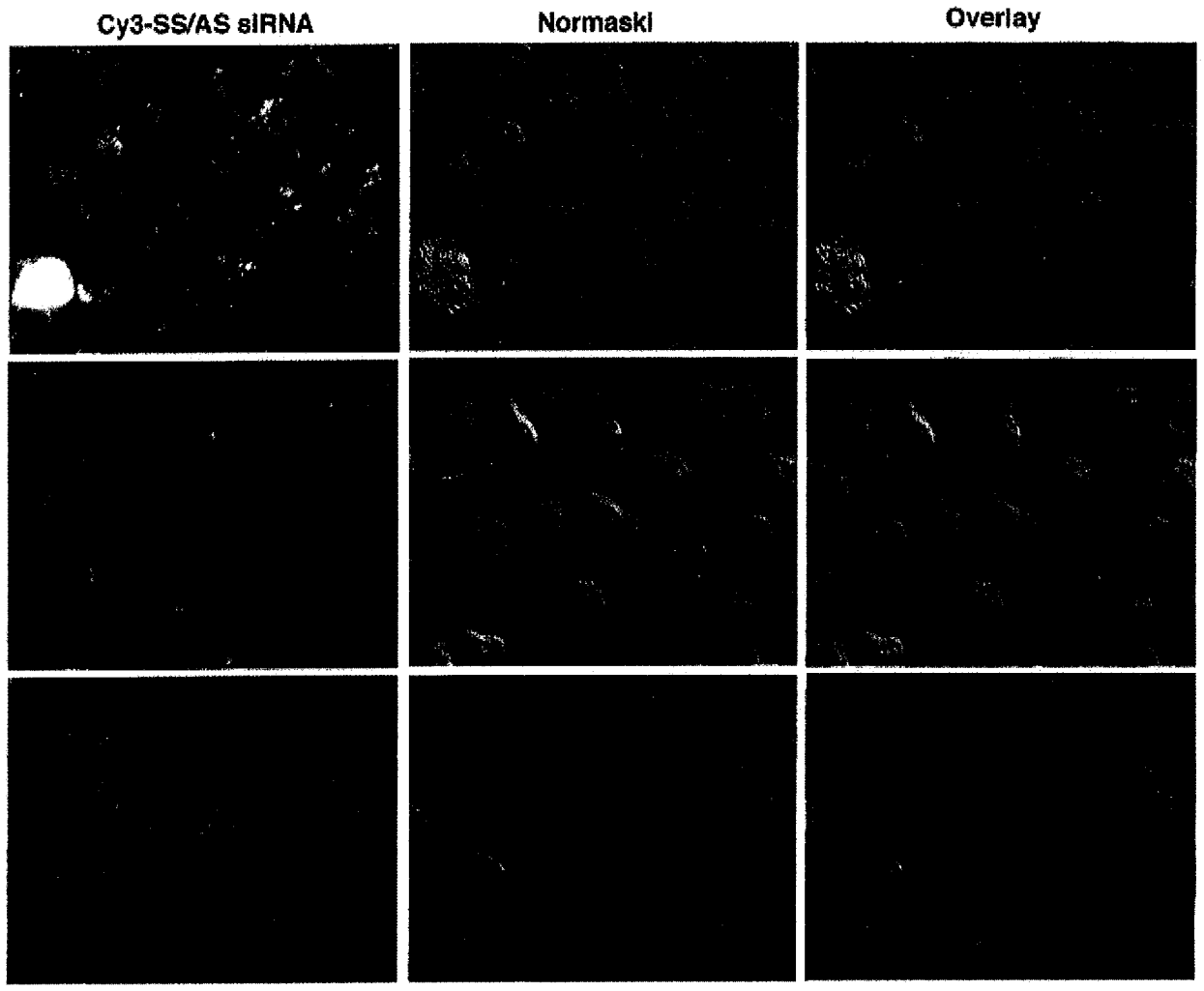
Dr

11/19/2002

Ya-Lin Chiu

11/19/02

Uptake of siRNA by HeLa Cells (40ug/1mL PAMAM-Mediated Transfection, 6h)(111902)



400X, 35 mm dish with coverslip bottom

Leico Demo

EXHIBIT 11

Th

11/19/2002
✓